Yeast display evolution of novel kinetically efficient peptide substrates for lipoic acid ligase

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***Short Abstract* — Fluorescence imaging is a powerful method to study protein dynamics in living cells, but existing probes are bulky (e.g., GFP), or insufficiently specific (e.g., FlAsH). We have developed new protein labeling methodology based on *E. coli* lipoic acid ligase (LplA), which attaches fluorophores and other probes to recombinant proteins fused to a 22-aa sequence (LAP1) (1). In previous work, we found that the slow kinetics of LAP1 prevented labeling of low or even medium-copy proteins. Here, we have used yeast display evolution to dramatically improve the kinetics of LAP, producing a new 13-aa LAP2 with kcat/Km of 0.98 μM^{-1} min^{-1}, compared to est. ~0.024 μM^{-1} min^{-1} for the previous LAP1. The superior kinetic properties of LAP2 permitted labeling of cell surface receptors with quantum dots.**

***Keywords* — Yeast display, peptide evolution, fluorophore ligase, quantum dots, protein labeling.**

I. Background and Summary

Most proteins are evolved to interact with a multitude of cellular molecules and thus contain several domains, binding sites, and activities. Often, it is useful to the biochemist to reduce a specific aspect of a proteins' function to just a peptide fragment. Our lab is interested in protein minimization to peptides, in order to develop protein labeling technologies. Size minimization of protein tags that direct the targeting of fluorescent probes (2) can greatly reduce problems of tag interference with protein trafficking, folding, and interactions.

In this study, our goal was to identify novel, kinetically efficient peptide substrates for *E. coli* lipoic acid ligase (LplA). LplA is a cofactor ligase that our lab has harnessed for fluorescent protein labeling applications (1). The natural function of LplA is to catalyze ATP-dependent, covalent ligation of lipoic acid onto specific lysine side chains of three *E. coli* proteins involved in oxidative metabolism: dihydrolipoyl acyltransferase (E2p, E2o)and glycine cleavage system (*H-protein*) (3). Previously, we showed that LplA and engineered variants could ligate unnatural probes such as alkyl azide (a functional group handle for fluorophore introduction;(1) and fluorinated aryl azide (a photocrosslinker) (4) in place of lipoic acid. To utilize these ligation reactions for protein imaging applications, we prepared recombinant fusions of proteins of interest (POIs) to the 9 kD E2p domain of pyruvate dehydrogenase (1). Such fusions could be labeled with high efficiency and specificity by our unnatural probes on the surface and in the cytosol of living mammalian cells (1)(unpublished).

Even though 9 kD (85 amino acids) E2p is considerably smaller than Green Fluorescent Protein (35 kD) and other protein labeling tags such as HaloTag (33 kD) (5) and SNAP tag (20 kD) (6), we wanted to further reduce its size, to minimize steric interference with POI function. We previously attempted this by rational design of an "LplA acceptor peptide" (LAP1) (1), based mostly on the sequence of natural substrate E2o, with a few additional rational mutations. LAP1 is 17 amino acids long, or 22 amino acids with the recommended linker. We found that LAP1 fusion proteins could be ligated by LplA to some probes (lipoic acid(1), alkyl azide(1), and aryl azide (4) *in vitro* and in cell lysate, but not on the cell surface except when the POI was very highly over-expressed (1).

By careful library design, tuning of selection conditions with the help of a model selection, four rounds of selection with decreasing LplA concentrations, and additional rational mutagenesis, we engineered a 13-amino acid LAP2 with a k_{cat} of 0.22 ± 0.01 sec^{-1} and K_m of 13.3 ± 1.78 μM (with lipoic acid). The catalytic efficiency (k_{cat}/K_m=0.98 μM^{-1} min^{-1}) is similar to that of E2p (kcat =0.24 sec^{-1}, Km = 1.2 μM , and k_{cat}/K_m=12 μM^{-1} min^{-1}), and far improved over LAP1 (kcat = 0.1 sec^{-1}, Km ~ 250 μM , and k_{cat}/K_m=0.024 μM^{-1} min^{-1}) (1). As a consequence of this improvement, we could easily lipoylate cell surface LAP2 fusion proteins, even at low POI expression levels. We also performed LplA-mediated specific quantum dot targeting to LAP2-LDL receptor. In comparison, quantum dot labeling was undetectable using the same receptor fused to LAP1.

References

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